EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Neutralization Confirmation Procedure for Products Evaluated with the AOAC Sporicidal Activity Test (*Bacillus* Species)

SOP Number: MB-12-01

Date Revised: 04-25-05

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Controlled Copy No.: _____

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1.0 SCOPE AND APPLICATION:

1.1 The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in efficacy testing. A neutralizing agent is used to inactivate the product's active ingredients, a process essential to achieving the desired contact time. Bacteriostatic activity may bias the outcome of an efficacy evaluation.

1.2 This SOP describes methodology which will be used to determine the effectiveness of neutralizers specified for sporicidal activity testing. Neutralization Confirmation Procedure is a carrier-based method (sterile carriers) which simulates the test conditions, but is designed to quantitatively assess the effectiveness of neutralizers across a broad range of spore concentrations.

1.3 In most cases, *Bacillus subtilis* (ATCC #19659) will be the test microbe selected for sporicidal testing, and will be used in the neutralization testing; however, if requested, other *Bacillus* species may also be used.

1.4 This method can also be used to determine the effectiveness of an alternative neutralizer, one not specified in the test parameters.

1.5 It is preferable to perform the neutralization assay concurrently with product testing; however, an independent, stand-alone assay may also be performed.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 AOAC SAT = AOAC Sporicidal Activity Test

2.3 CFU = Colony Forming Unit

2.4 PBDW = Phosphate Buffered Dilution Water

2.5 TSA = Tryptic Soy Agar

2.6 FTM = Fluid Thioglycollate Medium

2.7 DI = Deionized Water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism (*Bacillus subtilis*) are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01, Biosafety in the Laboratory. Biosafety level 2 practices will be followed for tests involving *Bacillus subtilis*; however, the appropriate biosafety practices must be addressed for individual microbes.

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant, prepare the disinfectant dilutions within three hours of the disinfectant treatment step unless test parameters specify otherwise.

4.2 Strict adherence to the protocol is necessary for validity of test results.

4.3 Use aseptic procedures for all test procedures involving manipulations of the test organisms and associated test components.

5.0 INTERFERENCES:

5.1 For each neutralizer and medium tested per study, one batch (preparation) should be used for all treatment and control groups. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS: None

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Calibration of equipment used in testing will follow the procedures and schedules outlined in the Laboratory's Standard Operating Procedures for Equipment. These include:

SOP EQ-01	Calibration and Maintenance of pH Meters
SOP EQ 02	Calibration of Thermometers
SOP EQ-03	Calibration and Maintenance of Weigh Balances
SOP EQ-05	Calibration and Maintenance of Timers
SOP EQ-08	Calibration and Maintenance of Automatic Media Dispensor

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 General Description of the Assay. The test conditions specified for product testing (e.g., H_2O hardness, Use Dilution, pH, Organic Soil, Neutralizer, Contact Time, Temperature) must also be followed for the neutralization confirmation assay.

This assay is designed to simulate the conditions of the AOAC SAT; however, sterile carriers are used instead of seeded carriers. Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (see Table 1). The inoculum is quantified by plating on a suitable agar such as TSA. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer x disinfectant interactions.

10.2 Preparation of Inoculum. The inoculum is serially diluted and applied directly to tubes of subculture media.

10.2.1 A concentrated spore preparation may be obtained from a commercial source such as from Presque Isle Cultures, 3804 West Lake Rd., P.O. Box

8191, Erie PA 16505.

10.2.2 Generating spores with amended nutrient agar. Prepare and harvest a test culture of *Bacillus subtilis* ATCC No. 16959 using nutrient agar amended with 5 µg/mL manganese sulfate according to the method described below. The spore suspension, once prepared, is titered, diluted to the appropriate concentration (approx. 1.0 x 10^8 CFU/mL) with sterile water.

10.2.2.1 Preparation of Test Culture:

a) A lyophilized culture of *B subtilis* is reconstituted with nutrient broth per instructions from ATCC (refer to SOP MB-02). A previously established stock culture of *B subtilis* may also be used.
b) Two additional daily (24 ± 2 hours) subcultures are made using 10 ml tubes of nutrient broth as the culture medium and the tubes are incubated at 37ºC on shaker at 150 rpm/minute.
c) This culture is used to inoculate the amended nutrient agar plates.

10.2.2.2 Inoculation of amended Nutrient Agar Plates:

a) Each plate is inoculated with 500 µL of *B. subtilis* culture. Spread the inoculum on each plate with a sterile bent glass or suitable sterile spreading device.
b) Wrap each plate with parafilm or place them in plastic bags. Incubate the plates inverted for 37ºC for 12-14 days.

10.2.2.3 Harvesting Spores:

a) To each plate add 10 mL cold sterile DI water and using an L-shaped spreader, remove the growth from the plate and pipet the suspension into 50 mL sterile conical tubes (10 plates = 8 tubes, ~ 12 mL each).
b) Centrifuge the tubes at 5000 rmp for 10 minutes @ room temperature. Remove and discard the supernatant.

c) Resuspend the pellet in each tube with 20 mL cold sterile DI water and centrifuge at 5000 rmp for 10 minutes. Remove and discard the supernatant.
d) Repeat twice.
e) Resuspend the pellet in each tube with 10 mL sterile DI water.
f) Store the suspension @ 5ºC.
g) The quality of spores can be assessed by examining the spore suspension with a phase contrast microscope or by staining.
h) The spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity.

10.2.3 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the spore suspension into 9 mL of PBDW or sterile deionized water. Three dilutions, (1×10^{-6}, 1×10^{-7}, and 1×10^{-8}) will be used to inoculate the neutralizer and subculture media tubes described below. The dilution series is based on an estimate of 10^8 spores per mL for the undiluted suspension. The target number of cells to be delivered is 5-100 CFUs/mL.

10.2.4 To estimate CFUs/mL, plate (pour plate or spread plate method) each of the three dilutions in duplicate on TSA agar. Briefly vortex each dilution tube prior to plating. See 10.2.7 for pour plate method and 10.2.8 for spread plate method.

10.2.5 If the product test conditions include the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum. Otherwise, the inoculum should be prepared without the addition of an organic soil load.

10.2.6 Record the dilution and plating information on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Serial Dilution/Plating Tracking Form and the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form (see 16.0).

10.2.7 Pour plate method: the TSA agar is prepared and tempered (approx. 1 hr) to 45-55ºC in a waterbath prior to use. Tempered TSA agar is added to the plate after the addition of the appropriate dilution, and swirled to spread the inoculum.

10.2.8 Spread plate method: allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, tilt the plate back and forth or, if necessary, use a glass spreading rod and plate spinner.

10.2.9 Incubate plates at 37±1ºC for 24-48 hours. Count colonies with aid of a plate counter. Colonies of *B. subtilis* are opaque, rough, dull, round irregular margins, and low convex. Colonial variation may be observed and is typical for this strain. Plates that have colony counts over 300 can be estimated or labeled TNTC. Record the counts on the Inoculum Enumeration Form for Neutralization Assay (see 16.0).

10.3 Product Sample Preparation.

10.3.1 Follow guidelines for product sample preparation provided by the sponsor for sporicidal activity testing.

10.4 Performing the Assay. The following instructions apply to the analysis of one neutralizer with one carrier type.

10.4.1 Each assay will require three sterile carriers. Use the carrier type required for the specific sporicidal test. Record the test information on the Information Sheet for the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) (see 16.0).

10.4.2 The product must be applied according to specific instruction provided in the test parameters. Expose each carrier to the disinfectant according to the contact time specified in the test parameters for efficacy testing.

10.4.3 Within 5 seconds, a set of 5 carriers is placed into one medication tube containing the disinfectant at time zero or 30 seconds. Transfer carriers according to the method specified in OPP SOP MB-15. Record the carrier transfer information on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test): Time Recording Sheet for Carrier Transfers (see 16.0).

10.4.4 Allow the 5 carriers to remain in the product per the specified contact time; however, only 3 will be used in the study.

10.4.5 After the last carrier of a set (5 total carriers) has been treated with the

disinfectant, and the contact time is complete, aseptically transfer carriers into tubes containing the specified neutralizer within 2 minutes. Transfer only 3 of 5 carriers, discard the remaining 2 carriers. Transfer carriers according to the method specified in OPP SOP MB-15. This set of neutralizer tubes (3 total tubes) will represent the **Neutralizer-Primary Subculture** Treatment. Each tube will be inoculated with one mL of each of the three inoculum dilutions as indicated in Table 1.

10.4.6 Following the last carrier transfer into the neutralizer tube, transfer each carrier into a culture tube containing the secondary subculture medium (e.g., FTM). This portion of the assay is not timed, but the transfers should be made as soon as possible. This set of tubes (3 total tubes) will represent the **Secondary Subculture** Treatment. Each tube will be inoculated with one mL of each of the three inoculum dilutions as indicated in Table 1.

10.4.7 Inoculated Controls.

The **Neutralizer-Primary** Inoculated Control contains three tubes of fresh, unexposed neutralizer-primary media.

The **Secondary Subculture** Inoculated Control contains three tubes of secondary subculture media.

The preparation (media preparation number) of each medium must be the same as used in the treatments. Each tube will be inoculated with one of three inoculum dilutions as indicated in Table 1.

10.4.8 Uninoculated Controls.

Neutralizer-Primary and **Secondary Subculture** uninoculated Controls. One tube each of uninoculated neutralizer and secondary subculture media will be included in the test and incubated with the other tubes. Sterility of carriers must be confirmed either in-advance or concurrently with testing. It can be confirmed by adding the carrier to a tube of 10 mL fluid thioglycollate medium and incubating for 5-7 days.

10.4.9 Inoculating the Tubes. Inoculate each *inoculated* treatment and control tube with 1 mL of the diluted spore suspension as indicated in Table 1. Tubes are inoculated following the transfer of all carriers.

Table 1. Inoculation of Treatment and Control Groups with Three Dilutions of the Spore Suspension*

Neutralizer-Primary Subculture Treatment	Secondary Subculture Treatment (with Carrier)	Neutralizer-Primary Inoculated Control	Secondary Subculture Inoculated Control	Neutralizer-Primary & Secondary Subculture Uninoculated Controls
1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	**Not inoculated** 1 Tube of Neutralizer 1 Tube of Secondary Subculture Media

*1 x 10^{-6} through 1 x 10^{-8}; based on an approx. starting suspension of 10^{8} spores/mL

10.4.10 Incubate tubes 5-7 days at 37± 1ºC.

10.5 Results are recorded as + (growth) or 0 (no growth). Record results on Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form (see 16.0). Confirmation testing of the growth will be performed as listed in section 10.6.

10.6 Identification and Confirmation Testing.

10.6.1 A minimum of one positive tube per treatment and control, if available, should be confirmed using Gram staining and selective media. If further confirmation is deemed necessary (e.g., presence of contamination in the test system) Vitek analysis may also be used.

For each treatment and control group, select the tube with growth (inoculated with the dilution with fewest CFU/mL delivered) and conduct confirmation testing on a sample of the growth.

10.6.2 Gram stains are performed on smears taken from the positive culture tubes. In most cases, *B. subtilis* will be the test microbe. Gram stain for *B. subtilis* is gram positive rod. For the additional confirmatory tests, a loopful of broth from each selected culture tube is streaked on TSA and incubated for 24±2 hr at 37±1ºC. Growth on TSA is observed after 24 hours. Colonies of *B. subtilis* are opaque, rough, dull, round, irregular margins, and low convex. Colonial variation may be observed and is typical for this strain.

10.6.3 Record confirmation results on the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet (see 16.0).

10.7 Interpretation of Results.

10.7.1 Plate count data. The plate counts are an essential element of this assay. One of the three dilutions plated should provide counts within the target range, 5-100 CFUs/mL. Neutralizer and subculture media tubes inoculated from this dilution also received this low level of challenge, an aspect critical to the determination of neutralization effectiveness and bacteriostatic activity.

10.7.1.1 The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

10.7.2 Controls. Growth in the **Secondary Subculture** inoculated Control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.* Growth in the **Neutralizer-Primary** inoculated Control should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media.

There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

The **Neutralizer-Primary** and **Secondary Subculture** uninoculated Control tubes are used to determine sterility, and must show no growth for the test to be valid.

10.7.3 Treatments. The occurrence of growth in the **Neutralizer-Primary Subculture** and **Secondary Subculture** treatment tubes is used to assess the effectiveness of the neutralizer. The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high Level of inoculum (e.g., the dilution with plate counts which are too numerous t count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or*

neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the ***Secondary Subculture*** *treatment tubes which received lower levels of inoculum (e.g., 5-100 CFUs/mL).*

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in secure file cabinets in D217. Only authorized personnel have access to the secure files. Archived data is subject to OPP's official retention schedule.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

13.3 Appropriate quality checks will be performed per the Laboratory's SOPs. These include (see ref. 15.4):

SOP QC-01	Quality Assurance of Purified Water
SOP QC-02	Air/Surface Monitoring of Microbiological Laboratories
SOP QC-03	Glass Washing and Detergent Residue Test
SOP QC-06	Use and Maintenance of Biological Safety Cabinets
SOP QC-07	Monitoring of Water Temperature of Recirculating Chillers
SOP QC-08	Monitoring Temperature/Humidity of the Disinfectant Sample Storage Room
SOP QC-09	Establishment of Control Numbers a for Laboratory Supplies
SOP QC-10	Expiration Time and Examination of Media and Reagents
SOP QC-11	Performance Assessment and Sterility Verification of Prepared Media and Reagents
SOP QC-13	Performance Verification of Autoclaves
SOP QC-15	Media and Reagent Preparation: Assigning Prep and

Sterilization Run Numbers
SOP QC-17 VITEK: Quality Control Procedures

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious.

15.0 REFERENCES:

15.1 SOP MB-15. AOAC Sporicidal Activity Test (*Bacillus* species).

15.2 SOP MB-01. Biosafety in the Laboratory.

16.0 FORMS AND DATA SHEETS:

16.1 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Information Sheet

16.2 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form

16.3 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Time Recording Sheet for Carrier Transfers

16.4 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet

16.5 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Serial Dilution/Plating Tracking Form

16.6 Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form

Information Sheet for the Neutralization Confirmation Assay (AOAC Sporicidal Activity Test)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP(s)	
Product Name		Test Date	
Product Sample No.		Neutralizer	
Product Lot No.		Comments:	
Expiration Date			

TEST PARAMETERS/Confirmed by:_____			
Diluent	Specified	Diluent Used	Hardness/Date/Init.
[redacted]			/ /
Organic Soil	Specified	As Prepared/Date/Init.	
[redacted]			
Neutralizer	Specified		
[redacted]			
Temperature (°C)	Specified	Chiller Unit Display	Test Tube Waterbath
[redacted]		Before: After:	Before: After:
Contact Time (minutes)	Specified	As Tested	
[redacted]			
Carriers (Unseeded)		Control #	Preparation #
[redacted]	Type:		

TEST MICROBE INFORMATION/Confirmed by:_____	
Test Microbe	
Org. Control No.	

REGENT/MEDIA INFORMATION/Confirmed by_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Results Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments:	

TEST RESULTS*: Date Recorded/Initials:_____			
Treatments/Controls	Inoculum Dilutions		
	1×10^{-6}	1×10^{-7}	1×10^{-8}
Neutralizer-Primary Subculture Treatment			
Secondary Subculture Media Treatment (with Carrier)			
Neutralizer Inoculated Control			
Subculture Media Inoculated Control			
Neutralizer Uninoculated Control Tube			
Subculture Media Uninoculated Control Tube			
*+ = growth, 0 = no growth			

SUMMARY OF RESULTS: Date/Initials:_____	
Bacteriostatic Effect Observed?	Yes_____ No_____
Comments:	

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test): Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No(s).	
Organism(s)	
Neutralizer(s)	
Carrier Type	

Initials /date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time for carriers (into the disinfectant)		Carrier Drop End Time for carriers (into the neutralizer)		Carrier Transfer (into secondary media)
			Clock	Timer*	Clock	Timer	Start Time[1]
	1						
	2						
	3						
Comments:							

* = ±5 seconds
1= Taken from clock

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Carrier Type[3]	

Source: Tube/Plate ID	Date/ Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)[2]

1 Record Gram Stain results as GPC=gram positive cocci, GNR=gram negative rods, GPR=Gram positive rods.
2 Vitek Identification Number 3. Porcelain penicylinders (P) or Suture loops (SL)

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test)
Serial Dilution/Plating Tracking Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Neutralizer(s)	
Sample No.		Organism Control #	

Confirmed by:_____	Dilution Tube								
	1	2	3	4	5	6	7	8	9
Vol. In Dil. Tube prior to Addition									
Volume Added to Dil. Tube									
Overall Dilution in Dil. Tube									
Volume Plated									
Overall Dilution on Plate									
Number of Plates per Dilution									
Media Plated Onto									
Comments:									

REGENT/MEDIA INFORMATION/Confirmed by_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Neutralization Confirmation Assay (AOAC Sporicidal Activity Test) Inoculum Enumeration Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Organism	
Sample No.		Sample No.	

RESULTS: Date/Initials:_____			
Plating Method			
	CFU per Dilution Plate		Average CFU per mL
Dilution	Plate 1	Plate 2	
1×10^{-6}			
1×10^{-7}			
1×10^{-8}			
TNTC = Too Numerous To Count			
Comments:			

REGENT/MEDIA INFORMATION/Confirmed by_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.